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Chemical characterization and antioxidant properties of exopolysaccharides from mangrove filamentous fungi *Fusarium equiseti* ANP2

A.M.V.N. Prathyusha[a,1], G. Mohana Sheela[b,1], P.V. Bramhachari[a,1,*]

[a] Department of Biotechnology, Krishna University, Machilipatnam, AP, 52100, India
[b] Departmnt of Biotecnology, Vignan University, Vadlamudi, Guntur, AP, 522213, India

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ABSTRACT

A filamentous fungi *F. equiseti* strain ANP2 with good exopolysaccharide (EPS) yield, apparent viscosity was screened and isolated from Krishna estuarine mangrove sediments. Based on molecular characteristics the selected strain was confirmed as *F. equiseti,* yielded EPS (4.9 ± 0.25 g/L) and whole cell biomass (3.5 ± 0.2 g/L) in MSM during early stationary phase. The crude EPS was purified by chromatography of Q-Sepharose and Superdex-75, affording Ms and MF-1 fractions were obtained, respectively. Chemical analyses revealed that EPS is primarily composed of neutral sugars and proteins. FTIR-spectroscopy revealed presence of hydroxyl, carbonyl groups and glycosidic bonds which correspond to typical heteropolysaccharide. GC analysis demonstrated that MF-1 mainly consist of mannose (72.6%) and glucose (27.3%). Viscosity studies exhibited typical non-Newtonian pseudoplastic behaviour with high viscosity. The scavenging ability on hydroxyl radicals, indicate that MF-1-EPS has good antioxidant activity. Current study demonstrated that MF-1 differed from previously characterized *Fusarium* sp., and can serve as potential antioxidant.
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1. Introduction

Mangroves are highly productive ecosystems of tropical region, harbouring enormous varieties of marine microbes [1]. Marine fungi are second largest ecologically diverse microbial group contributing to mangrove ecosystem's food chain as decomposers and documented to be effective in producing chemically novel and biologically active compounds [2,3]. Nonetheless most of marine fungi are new and scantily characterized species in mangrove ecosystem [4]. Microbes such as fungi, bacteria and some cyanobacteria produce a high molecular weight extracellular polymeric substance (EPS) during their metabolic process [5]. The EPS serves in formation of biofilm, localization of biogeochemical processes in sediments and aggregates. Many marine microbial EPS and their protein complexes possess unique chemical compositions, unusual structures with various biological activities due to their unambiguous aquatic environment [6–8].

However the functional importance of fungi in marine ecosystem is even a topic of debate and analysis. Formerly, ecological significance of marine fungi was unrecognized even if the diverse eco-physiological groups form communities that firmly affect existence and primary yield of marine ecosystems [9]. Currently, fungal EPS compounds drew an additional attention, because EPS production is seasonal variation independent, recovery and purification is comparatively much easier than other genera and became valid substitute to EPS from plant and algal origin. Interest is mostly increasing towards microbes from extreme marine ecosystem (cold seeps, deep-sea hydrothermal vents, polar regions, coastal hot springs *etc.*) should signify a huge source of uncultivated and unidentified fungi [10]. Despite their potential bioactive role, rheological behaviour [11], stability at high temperatures [12], pH and high ionic concentrations, the microbial EPS have enormous applications in cosmetic, biopharmaceutical, food industries and oil recovery *etc.* [12,13]. Additionally microbial EPS in marine ecosystem involves in energy flow, nutrient recycling and synthesis of humic enzyme-EPS complexes [14].

The Krishna estuarine mangrove in the east coast of India, is third largest coastal ecosystem, connected to Machilipatnam bay on its north and to the Krishna River on its south. In view of their copious existence, it is of intrinsic interest to examine the EPS

* Corresponding author.
E-mail address: veerabramha@gmail.com (P.V. Bramhachari).
[1] All authors equally contributed and equal first author.

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production, biotechnological importance as well as their ecological role of mangrove fungi. There is a diminutive availability of literature on Krishna estuarine mangrove ecosystems. It is therefore necessary to comprehend the possible role of selected mangrove fungi in diverse biotechnological applications. Hitherto there have been several reports on biological activities from diverse fungi in marine environments *viz. Phoma herbarum* [15], *Keissleriella* sp. YS 4108 [16], *Aspergillus* sp. [17,18], *Cordyceps sinensis* [19], *Penicillium* sp. [8]. However, the biological properties of mangrove fungal EPS was not fully characterized. With today's attention towards new polymers and renewable sources, EPS from mangroves associated fungi may perhaps signify the potential resource for antioxidants. Therefore, the present investigation was intended to extract, purify and characterize an EPS from a mangrove filamentous fungus *Fusarium equiseti* with antioxidant activity.

2. Materials and methods

2.1. Strains and fermentation

The current study involved 32 fungal isolates screened from mangrove detritus and sediments of Krishna estuarine mangrove sediments falls under geographical location of Machilipatnam, Andhra Pradesh i.e; 16°10′ N to 16°17′N latitudes and longitudes 81°09′E to 81°13′E, respectively. Fungi were isolated and screened for viscous EPS production as per standard protocol [20] by parallel plate geometry (PP50, 50 mm diameter, 0.1 mm gap) using Anton Paar Physica MCR 301, USA. Among the screened isolates, *F. equiseti* strain ANP2 produced viscous EPS after ice-cold isopropanol precipitation. The fungal strain was characterized based on standard molecular identification techniques *viz.* 5.8S rRNA gene sequencing as *F. equiseti*. The voucher specimen of strain was preserved as glycerol stock in laboratory for future reference. *F. equiseti* ANP2 was cultured in liquid medium with Glucose (25 g/L), sea salt (20 g/L), yeast extract (10 g/L), peptone (5 g/L), KH_2PO_4 (1 g/L), $MgSO_4$ (0.5 g/L) at 25 °C in a rotary shaker at 150 rpm for 7 days. Two-day cultured fungi were used as inoculum.

2.2. Molecular identification of selected isolate ANP2

The reproductive structure of *F. equiseti* was examined under optical compound microscope and SEM. Final identification of fungi was done by 5.8 ribosomal RNA (rRNA) sequencing. Using standard protocol, fungal genomic DNA was extracted. The internal transcribed spacers (ITS) of 5.8S rRNA were amplified by polymerase chain reaction (PCR) employing universal primers of ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The PCR amplicon was gel extracted, purified and sequenced [21]. Primarily, sequences analysis was performed on NCBI (https://www.ncbi.nlm.nih.gov) using BLAST software (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLAST_SPEC=TargLociBlast) and submitted to GenBank. Ten FASTA sequences of *Fusarium* sps. including the strain ANP2 were selected and aligned using multiple alignment software program CLUSTALW (http://www.ebi.eic.uk/clustalw). Sequence data was analysed to find closest phylogenetic homologue of selected fungal strain. A bootstrapped phylogenetic tree was built using MEGA7.0 software with maximum likelihood method [22].

2.3. Growth and EPS production of strain ANP2

Mineral salts medium supplemented with 2.5% glucose and 0.5% peptone was used to determine growth characteristics of strain ANP2. The cultures were kept in agitation at 28 °C on a rotary shaker, with 150 rpm until the culture reached stationary phase for 6 days. The broth culture was centrifuged at 6,000×*g* for10 min. The mycelial biomass was harvested and dried at 60 °C for 24 h. Then dry weight (dw) of mycelium was determined [23].

2.4. Preparation of EPS

Fungal cultures grown for 7days (stationary phase) were centrifuged at 6000×*g* for 15 min. The supernatant was filtered through 0.45 μm filter and then concentrated twice using an ultrafiltration (Amicon filtration unit). To the concentrate 3 volumes of ice-cold isopropanol was added and left overnight for precipitation. The precipitated EPS was centrifuged at 6000×*g* for 20 min and lyophilized prior to storage at −20 °C. The precipitate was dialyzed (molecular cut-off of 3500) against double distilled water. The crude EPS obtained was totally soluble in distilled water. All further analyses were performed by solubilising crude EPS in Milli-Q ultrapure water (Millipore) [17,21].

2.5. Purification of EPS

Crude EPS can be stored at −20 °C for a maximum of 2 weeks until further analysis. Crude EPS was fractionated with a Q Sepharose Fast Flow column (300 × 30 mm) attached to an AKTA FPLC system and eluted with step-wise gradient of 0, 0.05 and 0.5 mol/L NaCl. Polysaccharide content of individual fractions was estimated by phenol–sulfuric acid assay [24]. Then after, fractions were eluted using distilled H_2O and 0.3 mol/L NaCl. Both the eluents were pooled, dialyzed against double distilled water. The dialysate was further purified using Superdex 75 column (70 × 2 cm) with 0.2 mol/L NH_4HCO_3 as eluent with 0.4 mL/min flow rate. Major polysaccharide fraction was pooled and freeze dried [11].

2.6. Composition analysis

The concentration of uronic acid, protein and carbohydrate in EPS was estimated by colorimetric methods. Total sugar and protein contents were approximated by Phenol sulfuric acid and Bradford assays by taking glucose and BSA as standards [24,25]. Uronic acid content was assayed using carbazole–sulfuric acid method [26] taking glucuronic acid as standard.

2.7. Rheological properties of EPS

Viscosity of EPS was determined by dissolving EPS in Milli Q water. Measurements are taken in an (Anton Paar Physica MCR 301, USA) using parallel plate geometry (PP50, 50 mm diameter, 0.1 mm gap) with increasing shear rate at different concentrations for 1%, 2%, 3%and 4% (w/v) EPS. The effects of shear rate on apparent viscosity solutions at 25 °C were detected. A shear ramp was carried out by measuring shear stress as a function of shear rate from 0.01 to 100s^{-1}. The rheological properties were estimated in triplicates [27].

2.8. Gas chromatography analysis

Five mg of EPS was dissolved in 2 mol/L TFA (trifluoroacetic acid) and kept in water bath at 100 °C for 6 h. After hydrolysis, the compound was co-distilled using methanol for removal of excess acid. Sugars were changed into their alditol acetates upon treatment of sample with pyridine (2 mL) and $(CH_3CO)_2O$ (1.5 mL). Alditol acetate derivatives formed were determined by a gas chromatography (Perkin-Elmer Clarus SQ 8 GCMS with auto

sampler) using RTX-5MS 30 M, 0.32 mm column [28]. Separation of alditol acetate mixture was done by flame ionization detector (FID), and O_2 free dry helium was used as carrier gas at a flow rate of 25–30 ml/min forming trimethylsilyl derivatives. This overall process was executed using following conditions: H_2: 1.5 mL/min; air: 200 mL/min; N_2: 1.5 mL/min; injection temperature: 250 °C; detector temperature: 250 °C; column temperature: 212 °C using myoinositol (1 mg/mL) as internal standard. Monosaccharide sugars were identified by comparing with reference sugars (L-fucose, L-rhamnose, D-mannose, D-glucose, D-galactose and L-arabinose, *N*-acetyl glucose amine, *N*-acetyl galactose amine).

2.9. FT-IR spectroscopy

The EPS was mixed with potassium bromide (KBr) powder, triturated and then made into a 1 mm pellets for Fourier-transform infrared (FT-IR) analysis at frequency range of 4000–400 cm^{-1}. FT-IR analysis was performed on Perkin Elmer-100 FT-IR spectrometer [29].

2.10. Antioxidant properties

2.10.1. Reducing power

The antioxidant potential of reductones was estimated on their competence to stop free radical chain by donation of a hydrogen atom. 0.5 mL MF-1 solution was mixed with 2.5 mL phosphate buffer saline (0.2 M, pH 6.6), 2.5 mL of (1%, w/v) $K_3Fe(CN)_6$. Incubate at 50 °C for 20 min, and then add 2.5 mL of TCA (10%, w/v). The mixture was then centrifuged at 2000×*g* for 10 min. To 0.5 mL of supernatant add 2.0 mL deionized water, 0.4 mL $FeCl_3$ (0.1%, w/v) and incubate for 10 min at RT. The intensity of a resultant Perl's Prussian blue color was the measure of EPS reducing potential from Fe^{3+} to Fe^{2+} was estimated by absorbance at 700 nm in spectrophotometer [30]. Ascorbic acid (Vc) and ammonium bicarbonate was used as a positive and negative control, respectively.

2.10.2. Determination of hydroxyl radical (•OH) scavenging activity

The •OH radical scavenging activity of EPS was estimated based on the report of Ye et al [5]. To 0.7 mL of EPS solution added 1.0 mL phosphate buffer saline (0.15 mM, pH 7.4), 0.1 mL safranin T (0.52 mg/mL), 1.0 mL EDTA–Fe(II) (6 mM), 0.8 mL H_2O_2 (6%, v/v) and incubated at 40 °C for 30 min. After incubation the color intensity was measured at 520 nm [5]. The change in absorbance of reaction mixture was an indicator for scavenging ability of EPS for hydroxyl radicals. Ascorbic acid (Vc) and ammonium bicarbonate were used as positive and negative control, respectively. The scavenging activity of hydroxyl radical was then calculated by equation mentioned below:

$$\text{Scavenging activity}(\%) = \left[\frac{(A_{\text{sample}} - A_{\text{blank}})}{(A - A_{\text{blank}})}\right] \times 100$$

Where A_{sample} is absorbance of sample with reagent mixture, A_{blank} is absorbance of reagent mixture without sample, and A is absorbance of reagent mixture without sample and H_2O_2.

2.11. Statistical analysis

All measurements were expressed as mean ± standard deviation (SD) with each experiment conducted in triplicate. SPSS11.0 software version was used for statistical analyses. The criterion for significance was set at $p < 0.05$.

3. Results and discussion

3.1. Isolation and identification of F. Equiseti ANP2

F. equiseti ANP2 appeared as a white mycelium on PDA plate with hyphae. Cultural and reproductive traits of ANP2 showed thick-walled macroconidia with strong dorsiventral curvatures and septae (5–7), with noticeable whip-like and a tapering apical cell. The mycelia initially appeared white, but produced brown pigment on aging. The 5.8 s rDNA gene sequence data of *F. equiseti* ANP2, was deposited as accession number KY560311 in GenBank. The phylogenetic history of strain ANP2 was established using neighbour joining method [31]. An ideal phylogenic tree with a sum of branch length = 0.2653 is constructed (Fig. 1). In phylogenetic tree related taxa were grouped together using bootstrap method (500 replicates), was demonstrated next to branches [32]. Maximum composite likelihood method was used to compute the evolutionary distances among taxa [33]. Phylogenetic analyses were performed in MEGA7 [20].

3.2. Biomass and EPS production in mycelial culture

EPS production and growth profile are illustrated in Fig. 2. EPS and biomass production in culture medium was insignificant for initial two days (accelerating growth phase), and notably increased between day 3 and day 5 (exponential growth phase). However, spiky increase and minor decrease in EPS and biomass production was observed during 3 to 5 days of growth and 5 to 8 days, respectively. It is noteworthy that, whole cell biomass and EPS production reached stationary phase after 6 days incubation (2.98 ± 0.045 g/L EPS). However, decrease in biomass production was observed on the onset of 7th day (3.4 ± 0.023) resulted into decline phase. The strain ANP2 showed maximum EPS (4.9 ± 0.25 g/L) and whole cell biomass (3.5 ± 0.2 g/L) production during early stationary phase. The results indicated that EPS production was closely correlated with mycelial biomass growth in most of culture period. In a recent report, Sun and group depicted very less quantity of EPS (0.83 g/L) production in marine fungus *Epicoccum nigrum* JJY-40 in 7 day culture [34]. *Pestalotiopsis* sp. BC55 produced highest Biomass and EPS (1.32 ± 0.045 gm/l EPS) after 4 days of fermentation. Interestingly the results shown in present study corroborated with EPS (4.320 ± 0.022 g/L) and biomass production from an endophytic fungus *Pestalotiopsis* sp. BC55 at optimised conditions [35]. But in present study the isolate produced relatively high quantity of EPS (4.97 g/L ± 0.25) than those from other reported marine fungi (34, 35).

3.3. Purification of EPS

The procedure for extraction and purification of EPS from mangrove filamentous fungi *F. equiseti* broth culture are shown in flow chart (Fig. 3). Interestingly, 4.97 g/L of crude EPS was extracted from ice cold isopropanol precipitation. The crude EPS obtained was applied to Q Sepharose Fast flow column and washed with 0.3 mol/L NaCl. The fractions exhibiting TCHO contents were pooled and were further purified by Superdex 75 column and a fraction MF-1 was obtained (Fig. 1B). MF-1 yielded from crude EPS was about 60%. Notably, the fraction MF-1 consist of 92.4% total sugars and significantly low protein content (0.94%).The crude EPS could be determined as a glycoprotein since EPS indicated the presence of sugars and proteins as followed by phenol–sulfuric acid and Bradford assay, respectively. In addition, in carbazole–

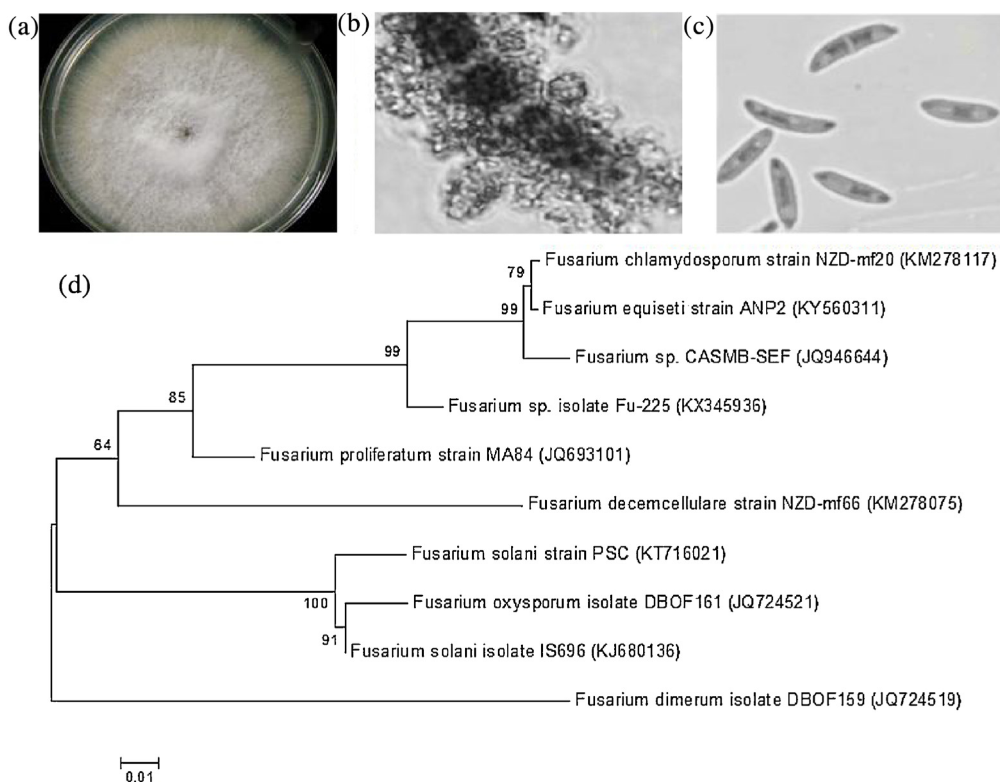

Fig. 1. (a) Colony morphology on PDA plate, (b) microscopic views of spore aggregation (c) microscopic views of individual spores of *F. equiseti* ANP2 using lactophenol blue staining (d) *F. equiseti* ANP2 and evolutionary relationships with some related fungi.

sulfuric acid assay no color was developed in reaction mixture method suggests that. EPS did not contain any uronic acid content.

3.4. Rheological properties

Fig. 4 depicts the flow curves of EPS with rising shearing rates at different concentrations. EPS solutions exhibited a typical non-Newtonian pseudoplastic or a strong shearing-thinning behaviour. Viscosity of polymer suspension raised with increase in EPS concentration conversely viscosity reduced when shear rate was augmented. This behaviour may be due to macromolecular network deformation in flow direction initiated by EPS shearing in solution. The viscosity was found to be 0.9, 1.2, 1.4, 1.7 Pa s^{-1} for concentrations 1%, 2%, 3%, 4% (w/v) of EPS, respectively at 0.01 s^{-1}

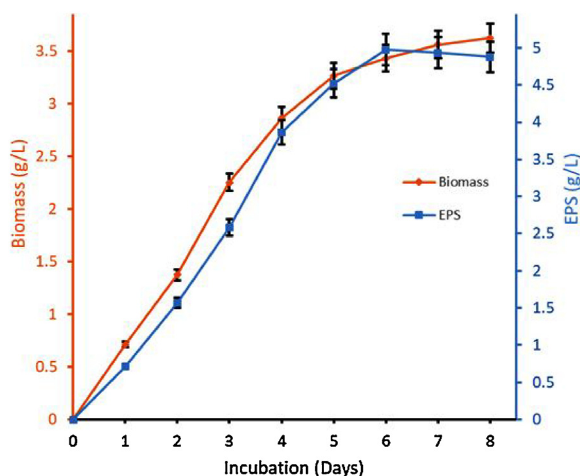

Fig. 2. Biomass and EPS production by *F. equiseti* strain ANP2.

shear rate [36]. The viscosity studies of isolate based on concentration reported that aqueous EPS solution had high viscosity and typical shearing-thinning behaviour (Fig. 4). The rheological analysis of *F.equiseti* EPS solution revealed its high viscous and pseudoplastic non-Newtonian fluid behaviour which implicates its potential as thickening and gelling agent in various industries. Rheological analysis helps in elucidating the structure-function relationship of the compounds at molecular level. In aqueous solvents the heteropolysaccharide shall prove to be beneficial in food, pharmaceutical industry (drug delivery) industry and as tissue engineering scaffolds [37].

3.5. Gas chromatography analysis

Gas chromatogram in Fig. 5 depicts the sugar composition of EPS. Different strains of *Fusarium* sp. were described to produce different EPSs. GC analysis demonstrated that MF-1 mainly consisted of mannose (70.6%) and glucose (25.3%). While minor components were fucose, rhamnose, xylose and arabinose. However, this is the first report on Krishna Delta Mangrove endophytic fungal EPS with mannoglucans as major polysaccharide constituents. The heteropolysaccharides from different mangrove fungus was reported earlier, such as EPS Fw-1 from mangrove associated fungus *F. oxysporum* was found to contain galactose, glucose and mannose [38]. The mangrove endophyte fungus *Aspergillus* sp. Y16 produces an EPS (As1-1) with galactose and mannose as monosaccharide sugar moieties [17]. The EPS (GW-12) produced by mangrove fungus *Penicillium solitum* consisted of Mannose [39].

3.6. FT-IR spectroscopy

FT-IR spectrum of MF-1 (Fig. 6) shows a strong peak at 3419 cm^{-1} represent oscillations of —OH groups present in

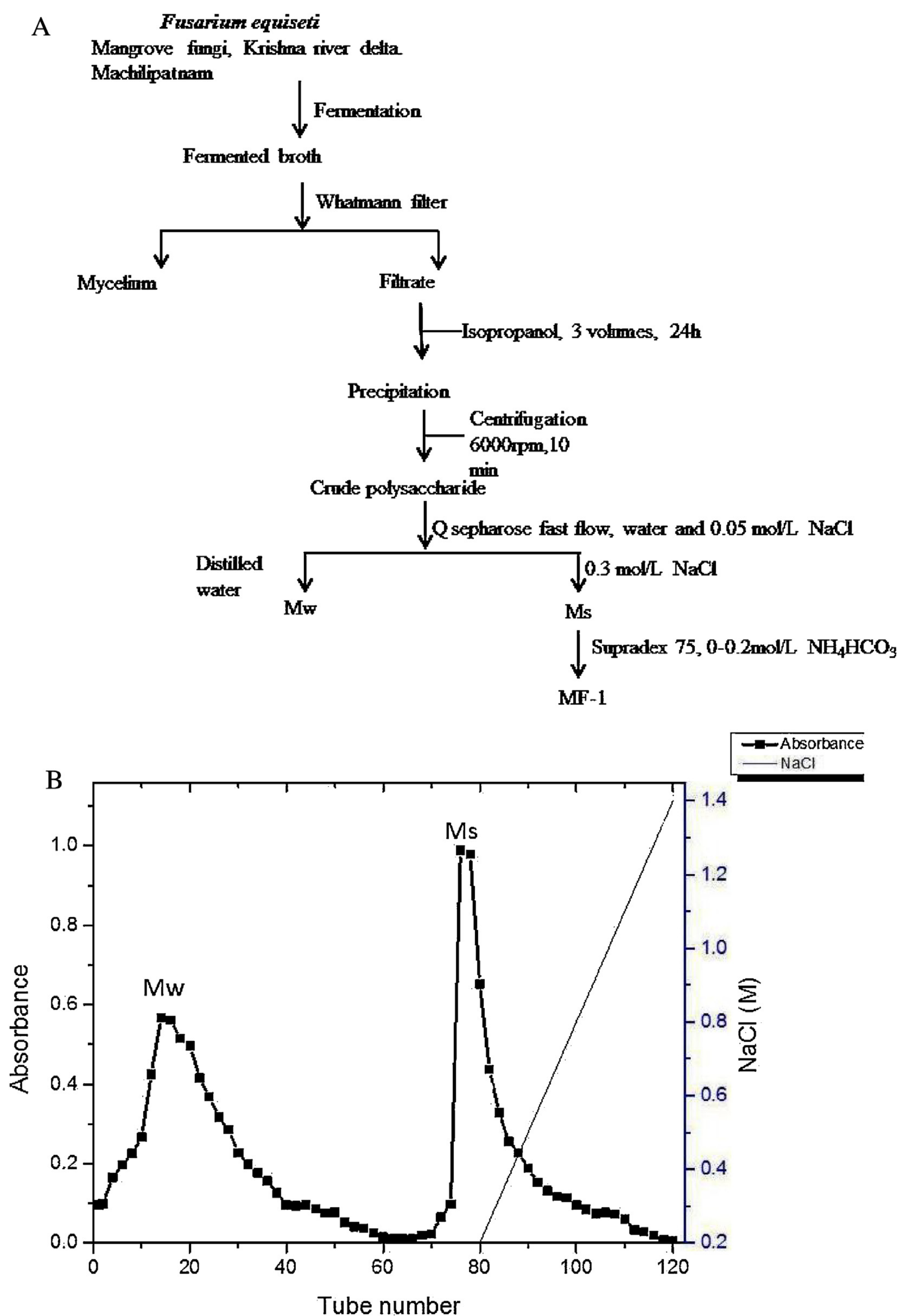

Fig. 3. A. Scheme for the extraction of the EPS produced by the mangrove filamentous fungus *F. equiseti* ANP2. B. The crude polysaccharides were applied to a Q Sepharose Fast Flow column. The fraction eluted with 0.3 mol/L NaCl was pooled and named as MF-1.

hydrogen bond of polymer. The peak at 2408 cm^{-1} was result of stretch oscillations of C—H bond. The stretching at 1636 cm^{-1} was assigned to oscillations of carbonyl bond C—O of amide group in protein or peptide and the signal at 1113 cm^{-1} assigned from bending oscillations of O—H bond. The signal at 985 cm^{-1} was obtained from C—O—C bonds vibration. The strong characteristic absorption at 852 cm^{-1} revealed the presence of α-anomeric [40,41]. The FTIR spectra of EPS reported the occurrence of carboxyl groups, hydroxyl, carbonyl groups and glycosidic bonds (Fig. 6). Carboxyl groups in EPS attacks on reactive site at divalent cations and exhibits sequestration. The presence of sugar moieties in fungal EPS indicates their effective application in fungal

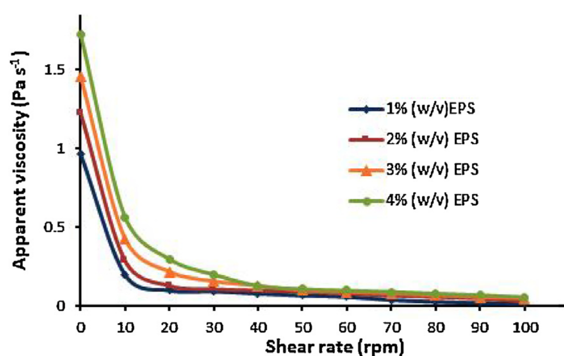

Fig. 4. The viscosity of EPS at different concentrations under different shear rates.

biosorption studies [42] and sequestration of heavy metals from mangrove estuarine waters.

3.7. Antioxidant activities *in vitro* of EPS

3.7.1. Reducing power of EPS

The reducing properties of reductones indicate their potential antioxidant activity. Reduction of Fe^{3+}/ferricyanide complex to its Fe^{2+} form is the measure of antioxidant potential of fungal EPS. Accordingly, Fe^{2+} formed was analysed by measuring the intensity of Perl's Prussian blue color at 700 nm. The reducing power of MF-1, using ascorbic acid, ammonium bicarbonate as standard was measured. The Fe^{2+} reducing powers of MF-1, ascorbic acid and ammonium bicarbonate increased with rise of concentrations. MF-1 reducing powers are less than ascorbic acid and noticeably higher

Fig. 5. (a) Gas chromatography profile of component sugars in EPS of standards *F. equiseti* strain ANP2. (b) Gas chromatography profile of standard sugars.

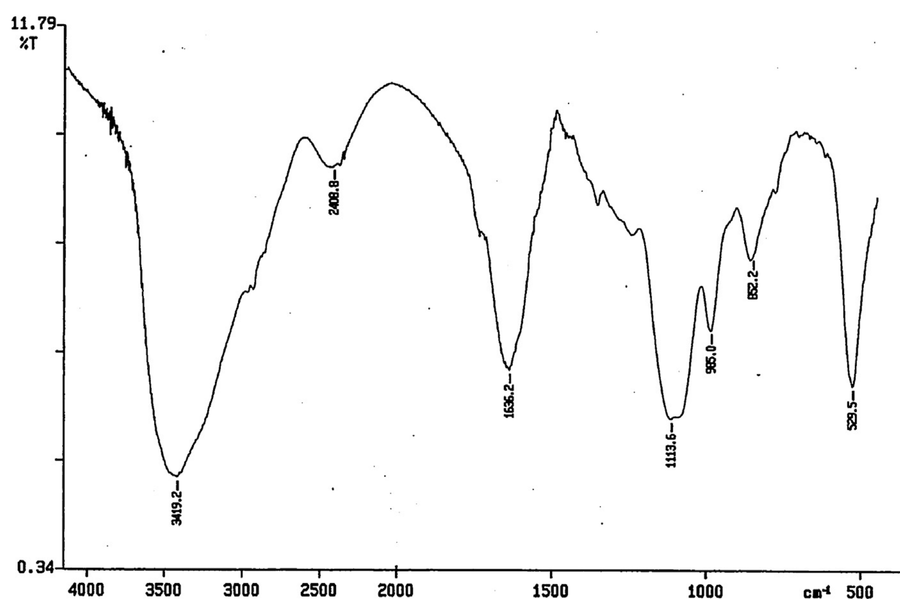

Fig. 6. FTIR spectrum of the extracellular polysaccharide MF-1 produced byF. *equiseti* strain ANP2.

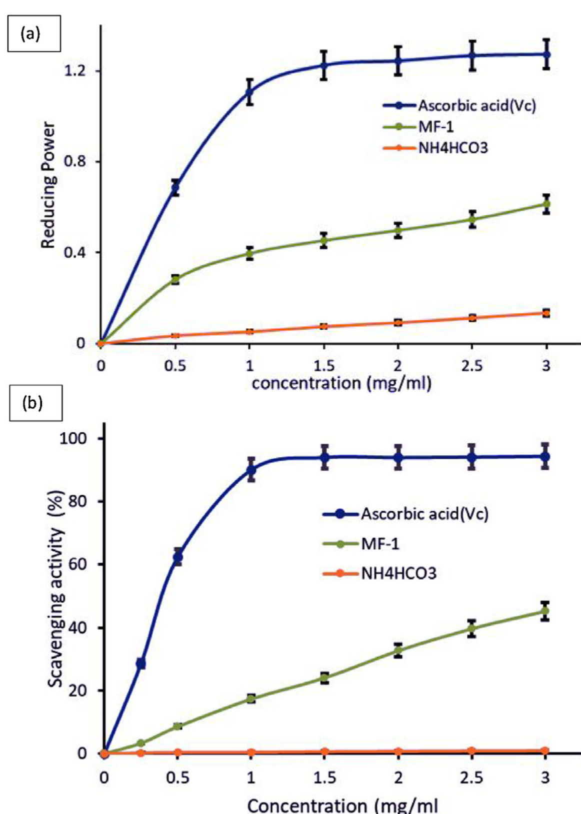

Fig. 7. Antioxidant activities of MF-1. Values are means ± SD of three separate experiments.
(a) Reducing power of MF-1 and ascorbic acid.
(b) Hydroxyl radical-scavenging activity of MF-1 and ascorbic acid.

than negative control ammonium bicarbonate. The results indicated that MF-1 has good reducing power (Fig. 7a).

3.7.2. Hydroxyl radical scavenging activity

Hydroxyl radicals are potent oxidants which can react with all biological macromolecules which damage living cells and are one of the significant free radicals [30] (Fig. 7b). Thus removal of hydroxyl radicals is an essential defence mechanism in cells. Hydroxyl radicals formed in reaction mixture, was rescued by MF-1. The scavenging effect of MF-1 for •OH was affected by concentration. Remarkably, at high concentration (3 mg/mL), the scavenging ability of MF-1, NH_4HCO_3 (negative control), Vc (positive control) were evaluated as 45.16%; and 1.15%, 94.35%, respectively. It is noteworthy that MF-1, showed high reducing power and free radicals scavenging activity. The scavenging activities of EPS might be due to hydroxyl groups of polysaccharides, which reduce free radicals to highly stable form or terminates free radical chain by donating electrons [11]. Many marine fungi, belong to genus *Pencillium, Aspergillus*, *Fusarium* could produce EPSs [18, 38, 39]. Chen et al. reported that the hydroxyl radical scavenging ability of an EPS obtained from *Aspergillus versicolor* LCJ-5-4 was about 49% at 4 mg/mL [18]. But in our study MF-1 reached 45% at a lower concentration level, 3 mg/mL. Thus, the EPS obtained from *F. equiseti* presented stronger antioxidant activities compared to *Aspergillus versicolor* LCJ-5-5 but lesser activity compared to mangrove fungus *Fusarium oxysporum* [38] (59% at a concentration of 2 mg/mL). In view of the complexity of antioxidant mechanisms, a broad understanding of the variation of monosaccharide composition, structure configuration and other structural characteristics of the EPS (MF-1) is warranted.

4. Conclusion

This is the first report on EPS isolation and chemical characterization from Krishna Delta Mangrove endophytic fungi, *F.equiseti*. The chemical composition analysis of polysaccharide secreted by ANP2 differed slightly from other *Fusarium* sp. MF-1 from *F. equiseti* strain ANP2 exhibited good *in vitro* scavenging abilities on hydroxyl radicals. The present study suggests that *F. equiseti* possess potential biological source of antioxidants with its structures worth being studied further. The results of study contribute significantly towards an understanding of the chemical composition of EPS and its biomedical applications. An in-depth examination of EPS with diverse structures may play a significant role in understanding relation between EPS and antioxidant activity mechanism.

Conflict of interest

We declare we don't have any conflict of interest.

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